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Naturally-occurring, Non-regressing Canine Oral Papillomavirus Infection: Host Immunity, Virus Characterisation and Experimental Infection

Philip K. Nicholls,*,†,[1] Brenda A. Klaunberg,‡ Richard A. Moore,† Elmer B. Santos,† Nigel R. Parry,§ Gerald W. Gough§ and Margaret A. Stanley†

**Department of Pathology and Infectious Diseases, Royal Veterinary College, Hatfield, Herts, AL9 7TA, U. K.; †Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, U. K.; ‡Arnold Pet Station, 1212 Ritchie Highway, Maryland 21012, U. S. A.; and §GlaxoWellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts, SG1 2NY, U. K.*

[1]To whom correspondence and reprint requests should be addressed. Fax: +44 (0)1707 661464. E-mail: pnicholl@rvc.ac.uk

Abstract

Papillomaviruses occasionally cause severe, non-regressing or recurrent infections in their human and animal hosts. The mechanisms underlying these atypical infections are not known. Canine oral papillomavirus (COPV) typically regresses spontaneously and is an important model of mucosal human papillomavirus infections. A severe, naturally-occurring, non-regressing, COPV infection provided an opportunity to investigate some aspects of viral pathogenicity and host immunity. In this case, the papillomas proved refractory to surgical and medical treatments, including autogenous vaccination and vaccination with capsid (L1) virus-like particles. High levels of induced anti-L1 antibodies appeared to have no effect on the infection. The papillomas spread to oesophageal mucosa, peri-oral haired skin, and remote cutaneous sites. Isolation of COPV from the animal and sequencing of several regions of the viral genome showed no differences to the COPV prototype. Experimental infection of beagle dogs with this viral isolate resulted in the uncomplicated development and regression of oral warts within the usual period, indicating that the virus was not an unusual pathogenic variant. These findings support the hypothesis that the recurrent lesions seen in some human papillomavirus infections, such as recurrent

laryngeal papillomatosis, are associated with specific defects in host immunity rather than variations in viral pathogenicity.

Key words: HPV, COPV, VLP, vaccine, immunity, recurrent papillomatosis

INTRODUCTION

The papillomaviruses are a ubiquitous group of pathogens infecting the skin and internal squamous mucosae of most vertebrate species, including man. These are classified by genotype and, to date, more than 80 types of human papillomavirus (HPV) have been isolated and sequenced (Delius *et al.*, 1998), with more than 130 types identified by detection of capsid (L1) encoding DNA (de Villers, 1997). The viruses are associated with a spectrum of epithelial atypia ranging from benign epithelial proliferations (warts) to cancer. Different viral types induce warts on skin, oro- and nasopharynx, larynx and anogenital tissue. The types infecting the anogenital tract have received considerable attention. Anogenital warts, induced mainly by HPV-6 and 11, are the commonest viral sexually-transmitted disease in the U. K. (Howett *et al.*, 1997). The erratic natural history of these lesions represents a significant therapeutic challenge. Infection with a subset of genital HPV types is strongly associated with the subsequent development of carcinoma of the uterine cervix and other anogenital malignancies in women, to the extent that HPV-16 and 18 have been defined as human carcinogens (Anon., 1996) with over 90% (Bosch *et al*, 1995) and maybe all (Walboomers and Meijer, 1997) cervical carcinoma tissues positive for HPV DNA. Papillomavirus infections therefore do not result in trivial, albeit unsightly sequelae, but are associated with life-threatening diseases. The development of strategies for the prevention or treatment of these infections is a high priority.

The development of such strategies requires an understanding of the host response to infection, which must encompass both innate and acquired immune defences. Papillomavirus infections result in lesions which persist for a variable period ranging from weeks to months or years. The infectious cycle of the virus is one in which viral replication, high level expression of viral proteins and viral assembly occur in terminally-differentiating epithelial cells remote from systemic immune defences and destined for death by apoptotic or similar mechanisms. The production of infectious virus is not accompanied by inflammation, and the activation of host defences is late. The importance of host immunity in the

control of papillomavirus infections is demonstrated in individuals immunocompromised either as a consequence of other diseases such as HIV or by therapeutic immunosuppression after organ transplantation (Palefsky *et al.*, 1998). These people exhibit persistent and florid HPV-induced lesions, refractory to therapy. However, even in immunocompetent individuals there is a spectrum of responses to infection, ranging from clearance of virus with no clinical disease through to persistent lesions refractory to all treatment modalities (Beutner and Ferenczy, 1997). This latter scenario is seen dramatically in individuals with recurrent respiratory papillomatosis (RRP). The general view is that individuals with persistent and recurrent florid infections of the type seen in RRP have an underlying and specific inability to mount a response to certain HPV types. However, it is possible that such infections are the consequence of infection with viral variants with an enhanced pathogenicity including immune evasion mechanisms.

Testing these hypotheses in humans is beset with ethical and logistic difficulties but natural papillomavirus infections in animals could illuminate these issues. Canine oral papillomavirus (COPV) infection is a valuable model for the analysis of many aspects of papillomavirus pathogenesis. COPV induces florid warts on the oral mucosa in domestic dogs and wild canids (for review see Nicholls and Stanley, 1999). Typically, COPV infection has an incubation period of 4-8 weeks followed by spontaneous immune-mediated regression within a further 4-8 weeks (Chambers and Evans, 1959). Persistence or neoplastic progression of COPV-induced papillomas has rarely been reported. In this report, we describe a case of naturally-occurring COPV infection in which the papillomas failed to regress and were refractory to all treatments, including vaccination. High levels of anti-L1 antibody were present throughout the period of investigation and after vaccination with L1 virus-like particles (VLPs) and an autogenous vaccine. Isolation of COPV from the animal and sequencing of several regions of the viral genome showed no differences to the COPV prototype. Experimental infection of beagle dogs with this viral isolate resulted in the uncomplicated development and regression of oral warts within the usual period, indicating that the virus was not an unusual pathogenic variant.

MATERIALS AND METHODS

Immunohistochemistry

Paraffin sections of formalin-fixed tissue were cut onto Vectabond coated slides (Vector Laboratories), dewaxed (3 x 5 min.) in xylene (BDH) and rehydrated through ethanol (BDH) dilutions (100%, 100% 95%, 70%, 30%, 3 min. each). After two 3-minute washes in PBS (phosphate buffered saline, Dept. of Pathology, University of Cambridge), endogenous peroxide was quenched for 30 min. in 0.3% hydrogen peroxide (Fisons) in PBS. After washing in PBS (3 x 3 min.), sections were incubated in normal goat serum (20% in PBS) for 30 min. at room temperature. The goat serum was blotted off and the primary antibody (CAMVIR-1 mouse IgG2a monoclonal, Dept. of Pathology, University of Cambridge. diluted 1:1000 in PBS) was applied overnight at 4 °C in a humid box. After washing as above, the secondary antibody (biotinylated polyclonal goat anti-mouse IgG2a, Amersham, 1:400 in PBS) was applied for 30 min. Slides were washed then incubated for 30 min. in peroxidase-conjugated avidin-biotin complex (Vectastain Elite, Vector Laboratories). Following washing, the chromogenic solution (0.06% 3,3' diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide in 0.1M Tris (BDH) pH 7.5) was applied and the reaction monitored by microscopy until completion. After washing, the sections were counterstained (Carazzi's haematoxylin, 45 seconds), dehydrated through graded alcohols and mounted in di-butyl-polystyrene-xylene (DPX, BDH). Positive control sections were canine oral papillomas with virions confirmed by transmission electron microscopy. Negative control sections were from normal canine oral mucosa.

DNA *in situ* hybridisation

Sections on Vectabond-coated slides (Vector Laboratories) were dewaxed and rehydrated as above and washed in PBS (2 x 3 min.). After protease K digestion in a humidified box (50 µg/ml in 0.02M Tris pH 7.4, 2mM $CaCl_2$ for 15 min. at 37 °C), sections were washed in PBS (2 x 5 min.), dehydrated through graded alcohols (30 s each) and air-dried. The section was covered in a digoxigenin-labelled, nick-translated (Boehringer-Ingelheim) genomic COPV DNA probe diluted 1:25 in hybridisation buffer (2 x SSC, 5% dextran sulphate, 0.2% Marvel (Premier Beverages), 50% deionised formamide), coverslipped and sealed with cow gum (Cow Proofings Ltd.). After denaturation on a hot plate (6 min. at 95 °C), slides were quenched in an ice bath then incubated overnight at 37 °C.

The gum was removed with forceps and the coverslips soaked off in 2 x SSC, 0.1% SDS. Slides were washed at room temperature in 0.5 x SSC (3 x 5 min.) at 55 °C in 50% formamide/1 x SSC for 10 min.,

and at 55 °C in 0.5 x SSC (3 x 5 min.). After 30 min. in 0.5% blocking buffer (Boehringer-Ingelheim), the sections were rinsed briefly in buffer 1 (0.1M Tris pH 7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05% Triton-X 100) and covered with 100 µl alkaline-phophatase conjugated anti-digoxigenin Fab fragments (Boehringer-Mannheim) 1:5000 in buffer 1 for 1 hour in a humid box. The slides were washed in buffer 1 (3 x 5 min.), equilibrated for 5 min. in buffer 3 (0.1M Tris pH 9.5, 0.1M NaCl, 50mM $MgCl_2$) then covered with 100 µl of 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim), 0.34 mg/ml nitroblue tetrazolium chloride (Boehringer-Mannheim) in buffer 3 and developed in the dark overnight. The reaction was stopped in TE buffer for 10 min. and the sections counterstained, washed in distilled water (2 x 5 min.) and mounted in Aquamount (Gurr).

Electron microscopy

Tissue cubes (1 mm) were dewaxed by three changes of xylene over 2 hours. After 15 min. in 100% ethanol, the samples were placed in 2% osmium tetroxide for 1 hour before being dehydrated via graded ethanols (40%, 70%, and 90%, for 10 min. each, followed by 100% for 3 x 15 min.). After submersion in propylene oxide (2 x 15 min.) the samples were placed overnight in a 1:1 mixture of propylene oxide and embedding resin (68% (v/v) dodecyl succinic anhydride, 32% (v/v) methyl nadic anhydride, 4% (v/v) dimethylaminomethyl phenol). The following morning the samples were placed into resin for 6 hours before embedding and baking overnight. Sections were examined under a Hitachi H-100 electron microscope.

ELISA

Flexible PVC microplates were coated with 100 ng per well of purified COPV particles suspended in carbonate buffer, pH 9.6, at 4 °C overnight. The plates were washed with PBS/0.05% Tween 20 and blotted dry prior to the addition of 100 µl per well of PBS/Tween containing 2% w/v skimmed milk powder (ELISA diluent) and incubation at 37 °C for 30 min.. Following further washing, 50 µl aliquots of individual serum dilutions, from a doubling series made for each dog serum sample, were added to duplicate wells of the coated plates. Negative controls received diluent alone. The plates were re-incubated at 37°C for 1.5 hours, washed again, and peroxidase-conjugated, rabbit anti-dog IgG (1/500, ICN immunobiologicals) was added to all wells. Following incubation for a further 1.5 hours and a final wash, the plates were developed using an s-phenylene diamine/peroxide substrate in urea buffer

(Sigmafast). Colour development was stopped with 20% sulphuric acid and absorbance levels were read at 490 nm using a Molecular Devices plate reader. The titre was expressed as the reciprocal of the dilution producing 50% of the maximum absorbance.

Viral isolation

Fresh frozen papillomas were diced finely and added to 15 ml PBS before Dounce homogenisation (Jencons). Both supernatant and debris were saved and processed separately.

The supernatant was poured off and centrifuged for 20 min. in a Sorval SS34 rotor at 7,500 rpm to pellet the nuclei. The pellet was resuspended in 4 ml of PBS and sonicated (Soniprep 150, MSE) on ice for 1 minute at power level 22. The lysate was layered onto 0.75 ml sucrose and centrifuged in an SW55Ti rotor at 34,000 rpm for 2 hours at 18 °C. The pellet was resuspended in 2 ml PBS before further sonication on ice for 10 seconds. Caesium chloride solution (1.92 g in 3 ml PBS, refractive index 1.381) was added and mixed by inversion. The refractive index was confirmed (Refractometer Model 60/ED, Bellingham and Stanley) as 1.365 (1.33 g/cm^3) before centrifugation in an SW55 Ti rotor at 34,000 rpm for 18 hours at 18 °C. The viral band was extracted and sealed in dialysis tubing (Medicell) and dialysed against PBS for four hours.

Viral challenge

Tenfold serial dilutions of the purified COPV isolate in a volume of 50 µl were injected superficially with a 25 g needle into the mucosa of the upper lip of beagles under general anaesthesia. The resulting vesicle or bleb was then punctured superficially 10 times with the same needle. Control sites received PBS instead of viral solution.

Viral PCR and sequencing

Primer pairs to regions within each of the viral open reading frames were designed and synthesised. To each 0.5 ml eppendorf tube was added 72 µl of distilled water, 2 µl dNTPs (10mM each of dATP, dGTP, dCTP, dTTP - Pharmacia), 5 µl of 5 µM Primer 1, 5 µl of 5 µM Primer 2, 5 µl (approx. 20 ng) of COPV DNA (extracted from fresh warts of the affected dog), 10 µl of 10x Taq buffer and 1 µl of Taq polymerase (both from Enzyme Technologies). In order to run all reactions together, a stepped annealing temperature was built into the cycle. Using a GeneAmp 9600 thermal cycler (Perkin-Elmer) a

5 min. 94 °C step was followed by 30 cycles of 94 °C, 53.4 °C, 57.1 °C, 58.1 °C, 63.6 °C, and 72.0 °C for 1 min. each, with a final annealing step of 72 °C for 5 min. before cooling to 4 °C. The products were analysed by 1% agarose gel electrophoresis alongside a 100 bp DNA ladder (Gibco).

RESULTS

A 3.5 year old neutered female labrador with severe oral papillomatosis (Fig. 1) was presented to one of the authors (BK). Diagnosis was established by histology of formalin-fixed paraffin-embedded tissue, immunohistochemical detection of the L1 viral capsid protein, *in situ* detection of COPV DNA (Fig. 2), and electron microscopic detection of viral particles (Fig. 3).

Removal of initial warts by electrocautery was followed in three weeks by appearance of a more abundant recurrent crop. Over the next eight months, further attempts at resolving the disease included electrocautery, surgical crushing of warts (to stimulate immunity), oral interferon-alpha, oral levamisole, intravenous immunoregulin, intravenous vincristine, subcutaneous vaccination with COPV L1-VLPs (five doses) and subcutaneous vaccination with an autogenous vaccine derived from homogenised warts (see Table 1). No significant response was seen and the dog was euthanased. Post-mortem examination revealed papillomas thoughout the oral cavity and extending down the oesophagus (Fig. 4). Papillomas were seen on the haired skin of the face, as well as more remote cutaneous sites such as the pinna and forelimbs. Examination of material removed post-mortem confirmed the presence of COPV in the lesions from oesophagus (Fig. 5), haired skin, and oral cavity. These data add oesophageal mucosa to the tissues known to be affected by COPV. Interestingly, a papilloma developed on peri-vulval skin but a biopsy was not available for confirmation of viral involvement.

No significant lymphocyte infiltrate was seen in biopsies of the papillomas. This is in contrast to the findings in warts regressing after experimental infection, in which numerous infiltrating $CD4^+$ and $CD8^+$ cells are present (manuscript in preparation). There was no haematological evidence of immunosuppression. The total white cell count was 7800/µl, at the low end of the normal range (8-18,000/µl). The neutrophil count was 5900/µl (normal range, 3-12,000/µl) and the lymphocyte count was 1900/µl (normal range, 1000-4800/µl). Serum protein electrophoresis revealed increased gamma globulins (1.28 g/dl, normal range of 0.4-1.0). Unfortunately, logistical factors prevented the

undertaking of lymphocyte functional assays, since the affected dog (USA) and the authors' laboratories (UK) were widely separated. No siblings of the affected dog were traceable, so a possible familial predisposition to florid papillomatosis could not be assessed.

To determine whether the severe clinical signs were due to an unusually pathogenic strain of COPV, the virus was isolated and injected into beagles. The lesions appeared and regressed normally. PCR amplification and sequencing of several regions of the isolated viral DNA (Table 2), including the entire L1 open reading frame, showed no differences from the COPV prototype sequence determined by Delius and others (1994). Additionally, positive immunohistochemistry findings (data not shown) with a COPV E4 antibody, raised against E4 derived from the prototype COPV sequence, further confirms the identity of this viral isolate, since E4 is highly divergent between papillomaviruses.

Serum samples were collected weekly during the period of vaccination with COPV L1 VLPs and autogenous vaccine. Sera were analysed by ELISA, using purified COPV virions as the antigen (Fig. 6). Sequential serum samples demonstrated an increase in specific antibody titre during the course of the infection. Vaccination with COPV VLPs or an autogenous wart homogenate was not associated with any clinical response, despite the presence of high antibody titres. The IgG antibody titres were greater than those from normal control dogs (mean titre of six normal beagles prior to infection with COPV).

Discussion

Various efforts have been made to demonstrate the influence of the immune system on the outcome of papillomavirus infection. Early studies failed to demonstrate increased persistence of papillomas in rabbits immunosuppressed using cortisone (Evans *et al.*, 1962). There are, however, occasional reports of severe or generalised papillomatosis in dogs immunosuppressed by prednisolone (Sundberg *et al.*, 1994; Nagata *et al.*, 1995; Le Net *et al.*, 1997). Oral papillomatosis has been seen also in a dog with hypogammaglobulinaemia (Bredal, 1996) and another report described multiple cutaneous squamous papillomas associated with IgM deficiency and impaired T-cell responses (Mill and Campbell, 1992). Studies in cattle support the hypothesis that immunosuppression after bracken feeding (Campo *et al.*, 1992; 1994) or azathioprine treatment (Campo *et al.*, 1992) worsens papillomavirus-associated disease, with lesions becoming extensive, extending down the oesophagus to the rumen without regression. The extension of non-regressing papillomas along the oesophagus in immunosuppressed cattle may be analagous to the findings in the dog reported here. Similar associations are documented in people

immunosuppressed by human immunodeficiency virus infection or iatrogenic means (Stark *et al.*, 1994; Benton and Arends, 1996). Occasionally, persistent and generalised human papillomavirus infections, refractory to treatment, have been associated with primary immunodeficiency (Gaspari *et al*., 1997).

The unusual natural COPV infection reported here provided an opportunity to investigate some aspects of non-regressing papillomatosis. The differential white cell count provided no haematological evidence of immunosuppression. The dog did not have signs of unusual or severe bacterial, protozoal or fungal infections, as might be expected in cell-mediated immunodeficiency. Serum protein electrophoresis demonstrated raised gamma globulin levels, probably due to increased immunoglobulins associated with an active B-cell immune response. ELISA demonstrated the presence of high titres of anti-COPV IgG. These findings indicate that successive crops of warts were able to occur despite the presence of neutralising antibody. Although the dog already had severe papillomatosis at first presentation, it is possible that the first episode of electrocautery may have allowed the virus to establish latency in numerous keratinocytes within the traumatised and regenerating epithelium. Latency has been demonstrated in other animal papillomavirus infections (Amtmann *et al*., 1984; Campo *et al*., 1994) and seems also to occur in RRP. Once latent infection was established, the presence of neutralising antibody would have been unable to prevent development of lesions from the infected keratinocytes. The numerous episodes of epithelial trauma, associated with five electrocautery sessions, may have caused reactivation of latent infections by the stimulation of epithelial proliferation and healing. Surgical interference has been documented as exacerbating papillomatosis in the dog (Collier and Collins, 1994). Although no published data address the issue of breed-related variations in response to papillomavirus infection, the authors (including a veterinary pathologist and a veterinary clinician) have seen multiple biopsies and clinical cases of uncomplicated wart regression in labrador dogs (unpublished observations) and are unaware of any such breed variations. These observations and the absence of significant lymphocyte infiltration in the clinical biopsies suggest that defective cellular immunity allowed warts to persist. Infection by a more pathogenic variant of COPV would be an alternative explanation. Sequence variants with differing pathogenicity have been described for some HPVs (Xi *et al*., 1997) and in the rabbit (Salmon *et al*., 1997). Infection with a more pathogenic variant of COPV was shown by sequencing and experimental infections not to be the cause of the unrestricted viral replication described in the present case.

The ELISA data from this unfortunate dog show that vaccination with VLPs, and increased antibody titres to the wild-type viral particle, failed to alter the course of an established infection, despite the titres being greater than those which would normally prevent re-infection. The development of high anti-capsid antibody titres is clearly ineffective in the removal of established papilloma infections, and design of immunotherapeutic strategies will need to take this into account.

The findings presented here document a new tropism for COPV, with viral infection not confined to the oral mucosa but extending throughout the oesophagus and also involving peri-oral haired skin and remote cutaneous sites. Additionally, these data show clearly that development of humoral immunity can neither clear established papillomavirus infections nor, under certain circumstances, prevent recurrent infections. Furthermore, these findings demonstrate that a virus normally causing insignificant spontaneously regressing papillomas can cause debilitating disease in some individuals. The most likely explanation of the phenomena in this animal is that host immunity was compromised. However, if this explanation is correct, the immune defect could be subtle, since the dog exhibited no other obvious signs of immune deficiency in either its haematological values or in its susceptibility to other opportunist pathogens.

These findings support the hypothesis that the recurrent lesions seen in some human papillomavirus infections, such as RRP, are associated with specific defects in host immunity rather than variations in viral pathogenicity. Additionally, persistent papillomavirus infections in some individuals are unlikely to be responsive to therapeutic vaccination, highlighting the need for a greater understanding of the biology of papillomavirus-associated diseases to facilitate development of non-immunological therapies.

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donated the COPV L1 VLPs used during vaccination. The protein electrophoresis was undertaken by Antech Diagnostics, Farmingdale, New York 11735. Initial diagnostic biopsies were read by Anne L. Kincaid (Antech) and Michael H. Goldschmidt of the University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania 19104.

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Table 1. Clinical Summary of a Case of Non-regressing Canine Papillomatosis

Treatment/ Clinical Observations	Dates
First presented for oral papillomatosis	1 Dec 1997
Surgical removal of oral papillomas, with electrocautery	4 Dec 1997, 22 Jan, 10 Apr, 30 Apr, and 3 Jun 1998
First recurrence of lesions	22 Dec 1997
Papillomas crushed with haemostats	29 Jan, 5 Feb, and 2 Apr 1998
Papillomas plucked with haemostats	11 Feb, 25 Mar, and 2 Apr 1998
Oral alpha-interferon (300 iu), once a day	11-17 and 24-30 Feb 1998
Oral levamisole (75 mg), every other day	2 Apr – 10 May, and 30 Jun-30 Aug 1998
Intravenous immunoregulin (1.5 cc)	10, 15, 18, 22, 30 Apr, and 3 Jun 1998
Oral enrofloxacin (100 mg) once a day	10 Apr – 5 May, and 3-10 Jun 1998
Intravenous vincristine (0.6mg)	8, 16, 22 May (all 0.6 mg), and 29 May 1998 (0.75 mg)
COPV L1 VLP vaccine (20 μg) with aluminium hydroxide, subcutaneously	6 Jun 1998
Booster COPV L1 VLP vaccine, without adjuvant, subcutaneously	12, 19, 25 Jun, and 2 Jul 1998
First dose of autogenous wart homogenate vaccine (1 cc), subcutaneously	2 Jul 1998
Booster autogenous wart homogenate vaccine, subcutaneously	9 (1 cc), 15 (1.5 cc), 22 (2 cc), and 29 (3 cc) July 1998
Euthanasia due to persistent papillomatosis of oral cavity, including larynx and peri-oral haired skin, and other cutaneous sites	6 Aug 1998

Table 2. PCR amplification and sequencing of purified COPV DNA isolated from a case of non-regressing papillomatosis

Primers		Predicted product	Size by electrophoresis	
Partial ORF series				
COPV E1	P1: caatc*gaattc*tgaggggcaaagacaggtag	330 bp including nt1199 to 1506	300-394 bp	(confirmed by sequencing)
	P2: taata*ggatcc*gcacaaacaaacataataat			
COPV E1b	P1: atcgta*ggatcc*aaatatcttcgcagcagatg	243 bp including nt2391 to 2609	Close to 234 bp	(confirmed by sequencing)
	P2: atcgta*gaattc*tcatacagactcagtagttcc			
COPV E2	P1: caaca*gaattc*aagggcataatgaggatagg	385 bp, including nt2694 to 3058	Close to 394 bp	(confirmed by sequencing)
	P2: attcg*ggatcc*ataatacacctgctgagtcc			
COPV E2b	P1: agctgg*ggatcc*taatagttt	259 bp including nt3460 to 3710	234-298 bp	(confirmed by sequencing)
	P2: atcgta*gaattc*ttataactcatctaaccctcc			
COPV E4	P1: cactt*gaattc*cggtgcctgttcccttaccc	257 bp including nt3193 to 3429	234-298 bp	(confirmed by sequencing)
	P2: atctt*ggatcc*cagaagtcttcccagtcgtc			
COPV E6	P1: caacg*gaattc*gatctttgcatgtctctaaa	363 bp including nt150 to 492	298-394 bp	(confirmed by sequencing)
	P2: atctc*ggatcc*ctgaccacagaaaaatcgtt			
COPV E7	P1: caact*gaattc*gagcagccggagccgataga	187 bp including nt575 to 740	154-234 bp	(confirmed by sequencing)
	P2: attcg*ggatcc*atcctctttttgagacagac			
COPV L1	P1: catcc*gaattc*atgtccaccaatagaactga	381 bp including nt7397 to 7755	Close to 394 bp	(confirmed by sequencing)
	P2: caact*ggatcc*gtccctcactagaaaccaga			
COPV L2	P1: caacg*gaattc*aagattaccactgatgctca	362 bp including nt5771 to 6110	298-394 bp	(confirmed by sequencing)
	P2: atctt*ggatcc*gctgatatcttcgcctatta			
Full ORF series				
COPV E1	P1: atcgta*ggatcc*atggcggctagaaaaggtact	1818 bp including nt816 to 2608	1.6-2.0 kb	(not sequenced)
	P2: atcgta*gaattc*tcatacagactcagtagttcc			
COPV E2	P1: atcgta*ggatcc*atggagaaactcagcgaggcc	1182 bp including nt2551 to 3708	1.0-1.6 kb	(not sequenced)
	P2: atcgta*gaattc*ttataactcatctaaccctcc			
COPV E7	P1: atcgta*ggatcc*atgattgggcaatgcgcaac	318 bp including nt533 to 826	Close to 344 bp	(not sequenced)
	P2: atcgta*gaattc*ctagccgccatggtcaagct			
COPV L1	P1: atcgta*ggatcc*atggcggtttggcttcctgc	1536 bp including nt6837 to 8348	Just under 1.6 kb	(confirmed by sequencing)
	P2: atcgta*gaattc*ttatttgcgtttgcgtttca			

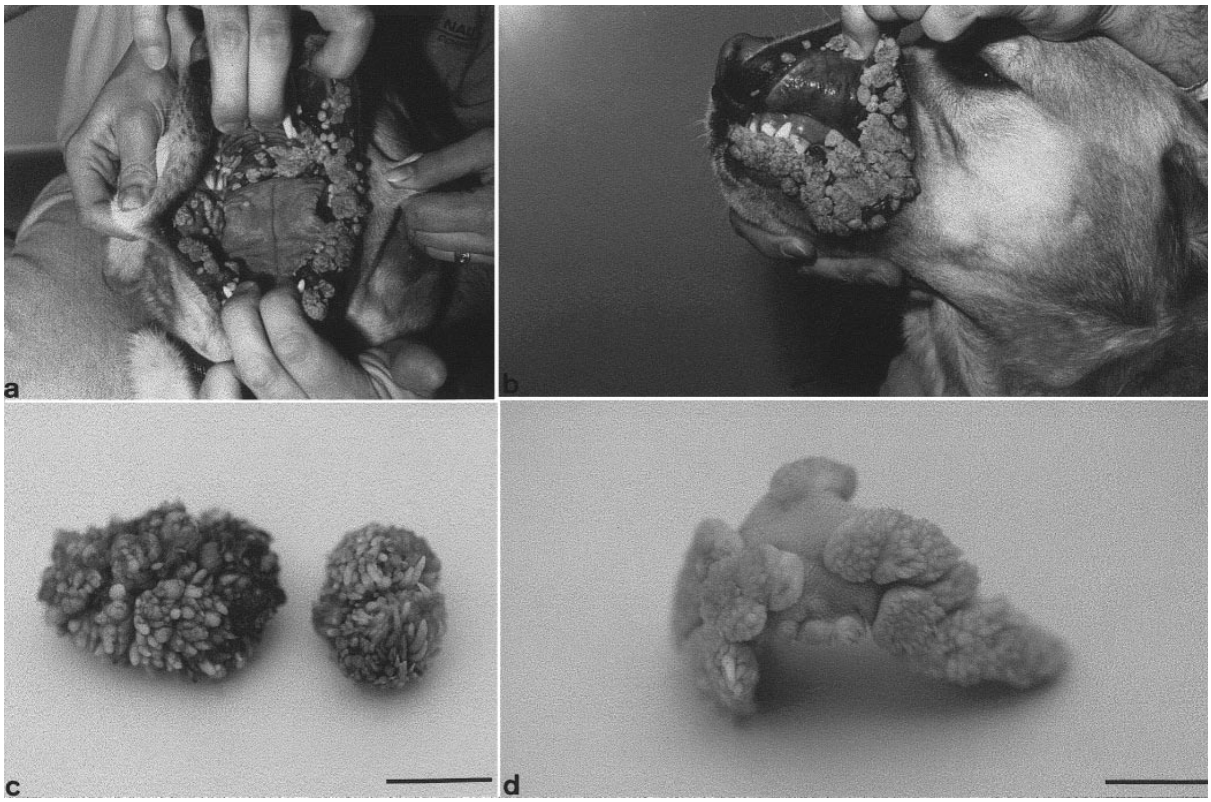

FIG. 1. Nonregressing canine oral papillomatosis. Multiple crops of papillomas affect the buccal mucosa, tongue, and palatine mucosa (a), extending onto the perioral haired skin (b). Papillomas of the buccal mucosa (c) and tongue (d) show multiple filiform papillae typical of mature warts. Bar, 1 cm.

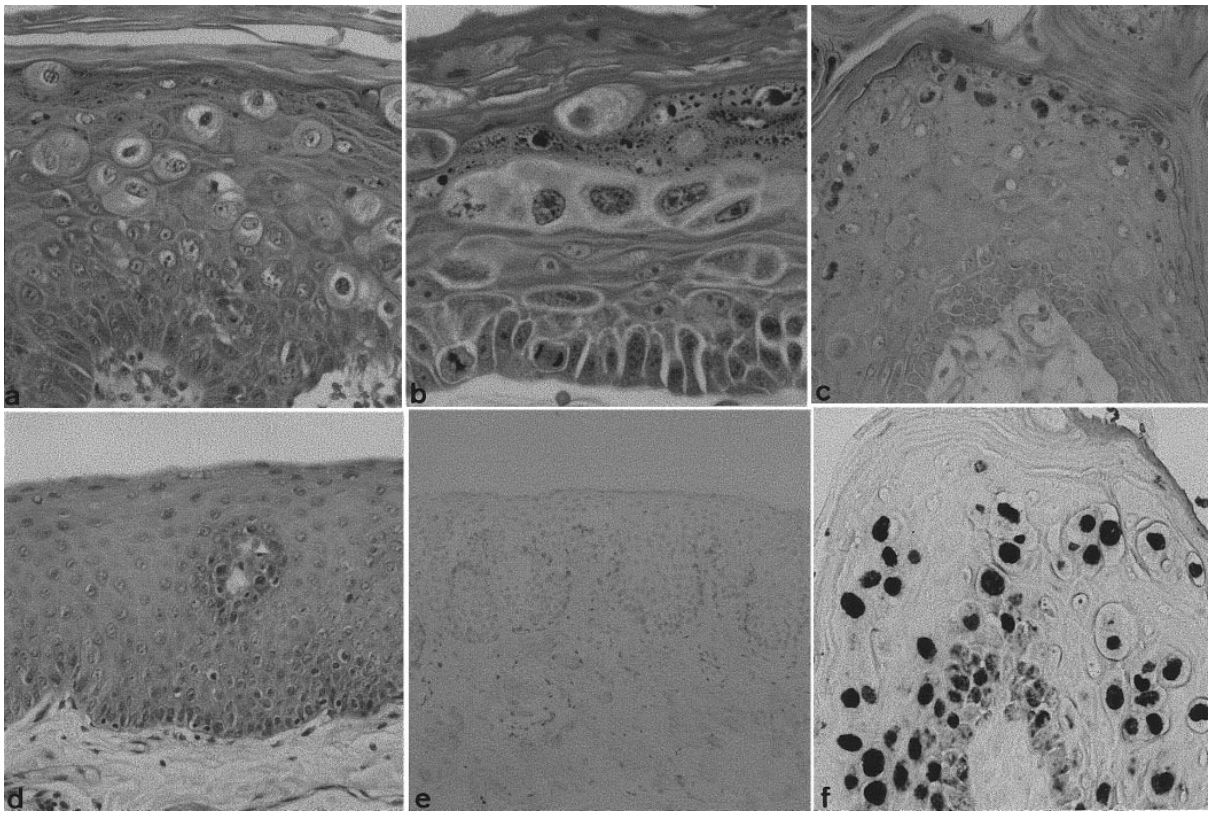

FIG. 2. Confirmation of diagnosis. Histological examination of haematoxylin and eosin-stained sections (a and b) reveals superficial vacuolated (koilocytic) keratinocytes typical of productive papillomavirus infections. Immunohistochemistry reveals abundant viral capsid antigen (L1) in the cells of the stratum granulosum (c). *In situ* hybridisation for COPV genomic DNA confirms the presence of abundant viral DNA in affected epithelial cells (f). Normal canine buccal mucosa (d) is included for comparison and is negative for viral DNA by *in situ* hybridisation (e). Original magnification: a, c, d, and f, 200×; b, 400×; and e, 100×.

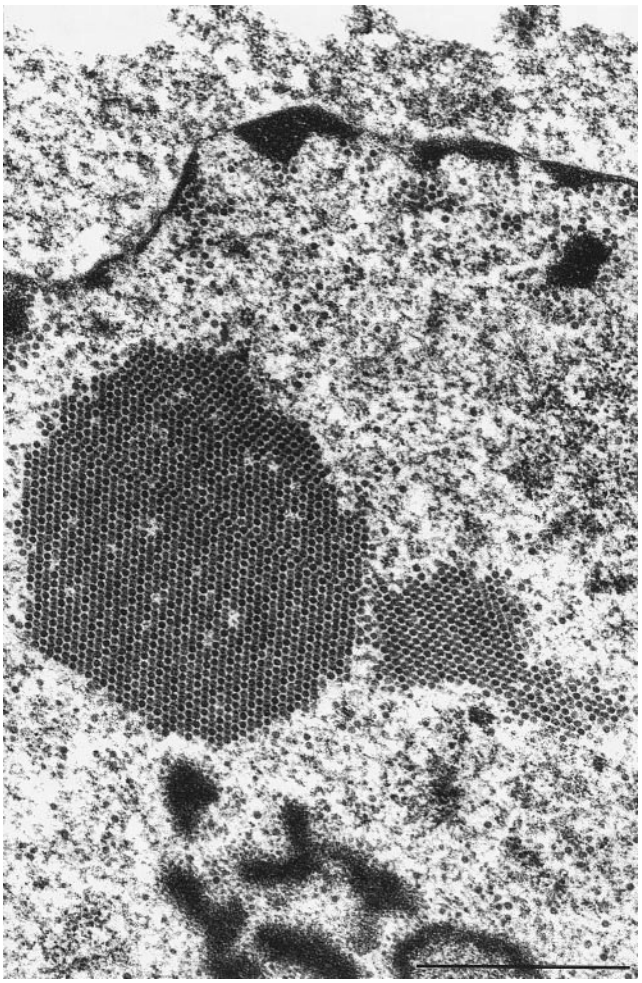

FIG. 3. Electron microscopy of canine oral papillomas. Abundant virions, both free and in close-packed crystalline arrays, are seen in the nucleus of superficial keratinocytes within the papillomas. The nuclear envelope is seen crossing toward the top of the picture. Bar, 1 mm.

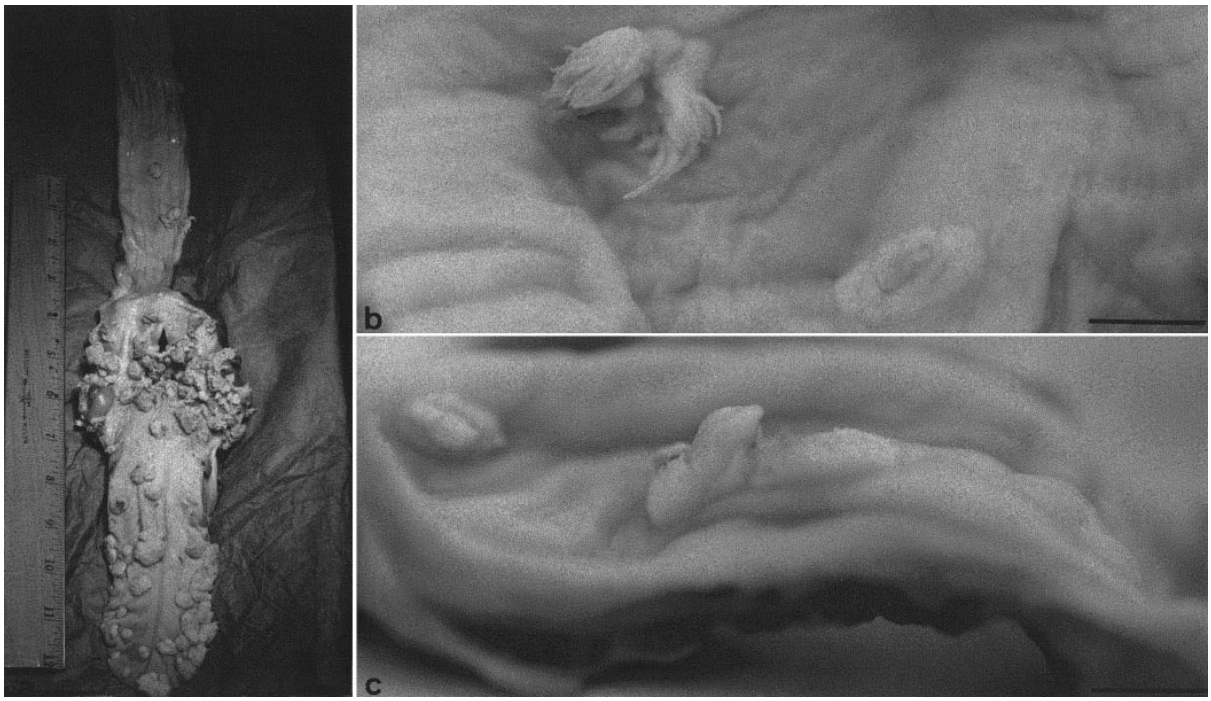

FIG. 4. Postmortem findings. At postmortem examination, papillomas were found to be widespread within the oral cavity, including the area immediately surrounding the larynx (a). Additionally, papillomas were found extending along the oesophagus (a–c). Bar, 1 cm.

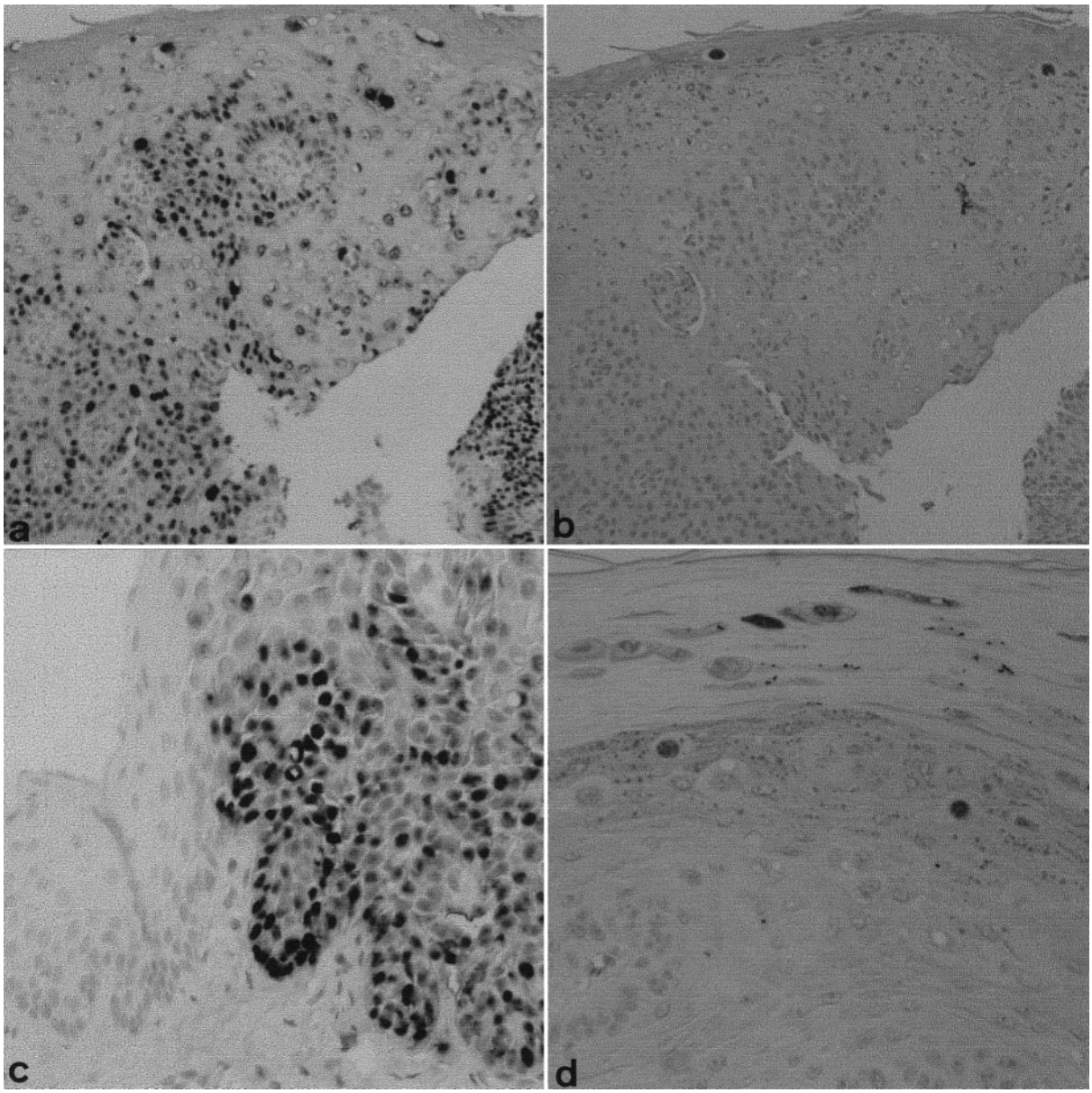

FIG. 5. Identification of viral DNA and capsid antigen in oesophageal lesions. Viral DNA was abundant within oesophageal papillomas (a and c), as detected by *in situ* hybridisation. Adjacent unaffected epithelium is free of detectable viral DNA, giving a sharp demarcation between negative (left) and positive (right) mucosa (c). Viral capsid (L1) antigen, detected by immunohistochemistry, was present in scattered superficial keratinocytes of the stratum granulosum and stratum corneum (b and d). Sections a and b are serially adjacent. Magnification: a and b, 100З; and c and d, 200З.

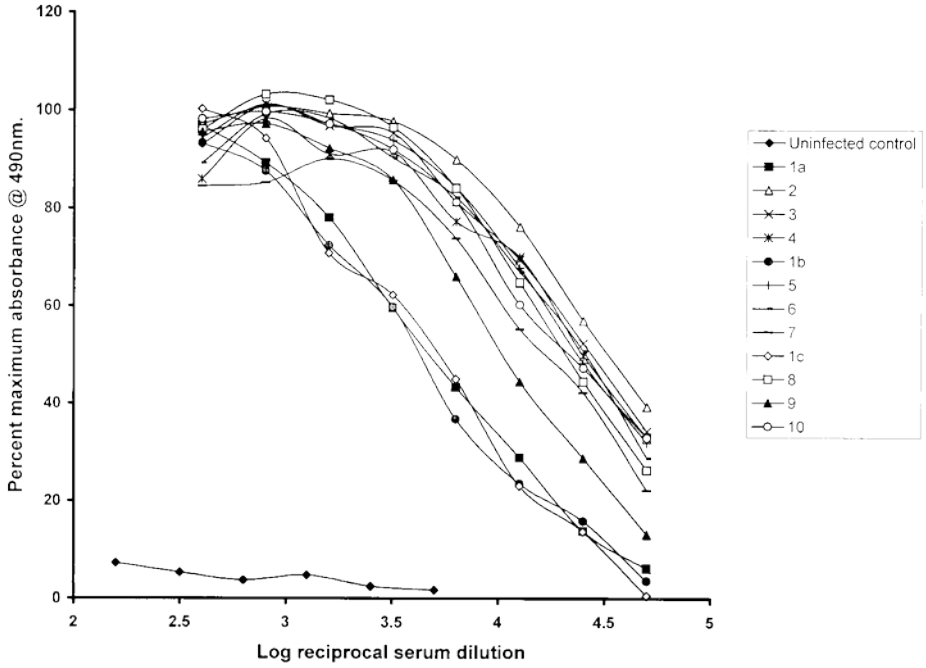

FIG. 6. Parallel ELISA titration of serum samples from a dog with nonregressing COPV lesions. Sera were taken before and during the period of vaccination with COPV L1 VLPs and autogenous wart homogenate. A rise in titer is seen during the course of the infection, but this failed to cause resolution of the lesions. The control data represent mean titres from six uninfected beagles.

Plate no.	Sample no.	Titre	Timing of sample	
1	1a	4,910	February 11, 1998	Prevaccination
1	2	33,350	June 6, 1998	1st dose VLPs
1	3	26,680	June 12, 1998	2nd dose VLPs
1	4	24,970	June 19, 1998	3rd dose VLPs
2	1b	4,450	February 11, 1998	Prevaccination (repeat sample)
2	5	24,325	June 25, 1998	4th dose VLPs
2	6	23,550	July 2, 1998	5th dose VLPs/1st autogenous vaccine
2	7	16,600	July 9, 1998	2nd autogenous vaccine
3	1c	4,870	February 11, 1998	Prevaccination (repeat sample)
3	8	21,160	July 15, 1998	3rd autogenous vaccine
3	9	11,840	July 22, 1998	4th autogenous vaccine
3	10	22,497	July 29, 1998	5th autogenous vaccine